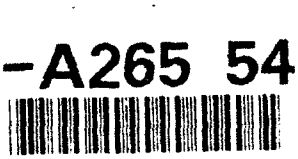

(2)

Project # N00014-91-C-0044

Brockbank, K.G.M.

Contract No. N000014-91-C-0044 Start Date: 31 JAN 1991

Cellular and Tissue Injury During Nonfreezing Cold Injury and Frostbite

Seventh Triannual Report: January - April, 1993

We have focused on four major areas of investigation:

1) the effects of cold-induced membrane phase changes on the function of transmembrane ion pumps;

2) the mechanism of metabolic inhibition under cold, acidotic conditions;

3) the influence of these conditions on the function of skeletal and smooth muscle; and

4) the role of free radicals in reperfusion injury in human cells.

The observations from these four areas of investigation are being used to develop methods for amelioration of cell and tissue damage during non-freezing cold injury and frostbite.

1. Impact of alterations in membrane fluidity on transmembrane ion pumps.

It is well established that lowering temperature leads to an increase in the viscosity of biological membranes. This temperature-induced change in membrane viscosity is associated with a phase change in membrane phospholipids from liquid crystalline to the gel state. These transitions have

been purported to induce concomitant decreases in the activity of transmembrane ion pumps, which can alter a cell's ability to maintain transmembrane ionic gradients and volume. For example, the cytosolic free calcium concentration is kept under strict control in cells by actively pumping calcium ions over a 100-fold gradient. High calcium levels can activate proteases and lipases, which could lead to cell destruction. However, the actual link between pump activity and membrane state has been the source of a great deal of controversy.

In this section of the research we determined the effects of hypothermia on both the phase properties of biological membranes and indirectly, by measurement of intracellular calcium concentrations, on transmembrane ionic pumps. For these studies we used human hemopoietic cell lines and human platelets as model systems.

We measured the membrane fluidity of whole cells by Fourier transform infrared spectroscopy (FTIR). FTIR is a non-invasive technique which requires no probe incorporation. We measured the thermotropic change in cellular membrane fluidity by monitoring the shift in the CH2 symmetric stretch peak (found at 2853 cm-1). CH2 bonds are located mainly in phospholipids. FTIR analysis of different human hemopoietic cell lines revealed an approximately 10°C range in membrane phase transition temperature.

The effects of 20 and 4°C incubations were examined to determine the role of the membrane phase transition in the chilling injury of cells. The hemopoietic cell lines were remarkably different in their sensitivity to hypothermia and there is an inverse correlation between cell survival at 4°C and the membrane phase transition temperature. Our studies to date have suggested that the membrane phase transition may play a vital role in cell sensitivity to hypothermia.

Further studies on the effects of hypothermia were undertaken using platelets.

Platelets were chilled to 20 or 4°C and then tested for spontaneous or agonist-induced aggregation. The data indicated that platelets chilled to 20°C aggregated spontaneously and were generally more susceptible to agonist-induced aggregation than either the 37°C controls or the 4°C chilled platelets. Platelets did not exhibit an increase in cytosolic free calcium or phospholipase A2 activity following platelet chilling. FTIR analysis revealed that the platelet membrane phase transition temperature is 20°C. Since the increased sensitivity of chilled platelets was not due to increased cytoplasmic free calcium or to phospholipase A2 activity, it is likely that it may have been induced by the relatively high membrane phase transition temperature.

We will continue to study the effects of chilling injury in platelets. The research will concentrate on making platelets less hypersensitive to agonists after chilling. The current hypothesis is that the platelet membrane is less fluid below normal body temperatures. As a result, platelets are more sensitive to agonist induced and spontaneous aggregation. This hypothesis will be tested by adding membrane fluidizers to chilled platelets and then testing the platelets for an aggregation response. Metabolic assays will also be undertaken to determine whether the fluidizers have any other effects on the platelets.

We have begun studying bovine aortic endothelial cells. We are using these cells as a model to develop protocols for human endothelial cell studies. We will be measuring the membrane phase transition, intracellular calcium concentration, and intracellular pH in endothelial cells. Increases in intracellular calcium concentration has been hypothesized as part of the mechanism of damage caused by various insults to cells, including hypothermia and hypoxia.

2. Metabolic transitions induced by local cold injury.

Cold-induced restriction of peripheral blood flow leads to anoxia in the affected region. Anaerobic glycolysis, the sole source of energy under these conditions, results in acidification due to the accumulation of lactic acid. The combination of cold and acidosis has been shown to inhibit glycolysis in certain systems. We proposed that during local cold injury, pH- and cold-induced inhibition of phosphofructokinase (PFK; the key regulatory enzyme of glycolysis) depresses metabolic flux and hence the amount of catabolic energy available for cellular maintenance. To test this hypothesis we initially studied human red blood cells, the results have been presented in prior interim reports.

We are now using our experience gained with red blood cells to study nucleated cells relevant to our future studies. Using these cells we will test whether stresses such as hypothermia, acidosis, and hypoxia can disrupt normal metabolism, either individually or in concert. There are several hypotheses being tested in these experiments. First, hypoxia should shift the burden of ATP production from the mitochondria to anaerobic processes such as glycolysis. Second, glycolysis in cells will be inhibited by low pH, even when the cell is dependent on glycolysis for ATP production. Third, at low temperature, the pH profile for activity of glycolysis and other pH sensitive processes will be shifted to a higher pH.

In order to test these hypotheses, we will measure heat production, adenylate energy charge, and intracellular pH measurements in endothelial cells after treatment with various stresses and combinations of stresses. We have begun this study using a bovine aortic endothelial cell line to develop the protocols. We have successfully measured heat production in the bovine endothelial cells by suspending then at high density in the presence of Percoll. Base line endothelial cell metabolism and the effects of acid pH have been studied. Three aspects of energy metabolism, metabolic heat production, lactate release, and

ATP levels were impaired by at least 42% at pH 6.3 relative to pH 7.4. It s likely that this peturbation of energy metabolism affects endothelial cell function and interactions with platelets and smooth muscle. Now that the methods for these studies have been established using bovine endothelial cells, we will switch to clinically more relevant human endothelial cell models. Endothelium plays an important role in regulating hemodynamics through interactions with both smooth muscle and platelets.

3. Impact of local cold injury on function of skeletal and smooth muscle.

All the biochemical and biophysical parameters described above can impact, either alone or in concert, on the physiological function of smooth and skeletal muscle. We have tested the influence of cold ischemia on the capacity of skeletal muscle fibers and vascular smooth muscle to contract and relax. Rabbit skeletal muscle fibers and isolated saphenous veins are being used as model systems.

We previously reported our studiesusing sarcoplasmic reticulum (SR) enriched membrane vesicles. SR vesicles turned out not to be a suitable preparation in which to study the P_i and temperature dependence of calcium uptake. Therefore, we have developed a saponin-skinned skeletal muscle preparation in which to carry out these experiments. Saponin skinning permeabilizes the sarcolemma but leaves the SR membrane intact. Calcium uptake and release can be measured from the amount of calcium remaining in the SR (measured by the contractile response to a high concentration of caffeine, which releases all calcium in the SR) without the need for bathing in an oxalate medium. We have initiated use of this model system to measure the effects of pH, P_i, ADP and temperature on calcium uptake and release from the SR. These experiments will provide information on how the stresses encountered during hypothermia and ischemia impact on the calcium sequestering activity of these

organelles. These studies will also provide information regarding the effects of fatigue upon tissues during or after hypothermic exposure.

We have initiated our studies of vascular smooth muscle survival and function during and after prolonged exposure to cold using a rabbit vein model. We have found that exposure of rabbit jugular veins to 20°C results in enhanced sensitivity to norepinephrine, the important circulating contraction-inducing hormone, and a reduction in sensitivity to the relaxing agent acetylcholine. These studies may imply that communication between vascular endothelium and smooth muscle is impaired by cold exposure. In addition, these observations confirm that the jugular vein model is appropriate for studies of hypothermia-induced vasoconstriction.

The objective of our studies during the remaining 8 months is to define the length of exposure to cold, anoxia, and acidosis, either separately or in combination, that results in irreversible smooth muscle function and the time course of recovery of veins subjected to shorter periods of insult. These studies will set the stage for definition, prevention, and intervention regarding the lesion(s) causing irreversible damage of smooth muscle and endothelium.

4. Role of free radicals in hypothermic injury in human tissues.

Oxygen free radical formation upon reperfusion of ischemic tissues and organs has been implicated in tissue damage in several animal models. However, the role of free radicals in human tissues is a matter of debate in the literature. As part of our studies characterizing the effects of cold induced injury in the hemopoietic cell lines, we noted that the addition of the membrane fluidizer α-tocopherol (vitamin E) increased the survival of cells incubated at 4°C. α-tocopherol is an oxygen scavenger and we now have evidence to demonstrate that the protective effect of this molecule is due to its activity as an oxygen

scavenger, not as a membrane fluidizer. Comparison of other antioxidants with α-tocopherol demonstrates that hydrophobic, but not hydrophilic, antioxidants are effective for extension of cell functions after prolonged exposure to cold.

During the remaining months of this project, we will test hydrophobic antioxidants on isolated and intact vascular tissues. The implications of cold protection by these compounds may be profound, since at least in the case of α-tocopherol treatment of military personnel prior to cold exposure could be performed without concerns about potential toxic side effects.

5. Amelioration of cell and tissue damage during non-freezing cold injury and frostbite.

As an initial entry into this area of research we investigated the feasibility of using antifreeze peptides to prevent frostbite damage due to ice crystal formation. Antifreeze proteins are found in the blood of Antarctic fish and are vital in the prevention of freezing injury in the fish by inhibiting the growth of ice crystals. We developed a model for analysis of recombinant antifreeze protein effects in a system when ice crystal growth can cause damage. We have reported our results in the red cell model in prior interim reports.

Further recombinant antifreeze peptide studies have been performed using slow cooling rates and nucleated human hemopoietic cells. Slow cooling rate conditions are closer to cooling rates expected during frostbite. These experiments have demonstrated negative dose dependent antifreeze peptide effects on cell survival during freezing and thawing. These observations suggest that it is extremely unlikely that this particular fish-derived antifreeze peptide may ameliorate or prevent the tissue damage associated with frostbite. However, there are other classes of antifreeze compounds found in cold hardy invertebrates which permit these organisms to survive freezing. These compounds would be

worthy of study, but are limited in their availability.